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(54) Title: ESSENTIAL OLIGONUCLEOTIDES OF VERTEBRATE TELOMERASE

(57) Abstract

DNA sequences encoding truncated RNA oligonucleotides of the vertebrate telomerase RNA component essential for the function of vertebrate telomerase are disclosed and their uses described. Vertebrate telomerase produced by combining an RNA oligonucleotide or an isolated RNA component with vertebrate telomerase protein is also disclosed.

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Essential Oligonucleotides of Vertebrate Telomerase

Related Applications

This application is a continuation-in-part of copending United States patent application Serial No. 08/478,352, filed on June 7, 1995. The teachings of this application are expressly incorporated herein by reference.

Background of the Invention

Telomerase is an enzyme essential for telomere length maintenance. Conventional DNA polymerases cannot complete the replication of chromosome ends and, without a mechanism to overcome this problem, chromosomes are predicted to shorten with each round of cell division. Watson, J.D. (1972) *Nature New Biol. 239*:197-201. Telomerase is a specialized telomere specific polymerase comprised of RNA and protein components, which elongate chromosomes through *de novo* nucleotide sequence addition.

A steady state equilibrium of telomere length is established in immortal single cell eukaryotes and is regulated by a number of different genes. Greider (1994) *Current Opinion in Genetics and Dev. 4*:203-211. Strikingly, length maintenance does not occur in primary human somatic cells and when they are passaged in culture, telomere length decreases in these cells. Primary human cells have a limited lifespan in culture and telomere shortening correlates well with loss of replicative capacity. Harley, *et al.* (1990) *Nature 345*:458-460; Allsopp, *et al.* (1992) *Proc. Natl. Acad. Sci. USA*, USA 89:10114-10118. Telomere shortening in tissues *in vivo* has been demonstrated for fibroblasts, leukocytes, and endothelial cells. Germ cell telomeres do not shorten with age, suggesting the germline is protected from telomere

loss.

Recent evidence suggests that the telomerase enzyme may be a new target for cancer therapy. Short telomeres are also found in cancer tissues. Telomere shortening may be due to the inability of conventional polymerase to replicate chromosome ends. Kim, *et al.* (1994) *Science 266*:2011-2015, were not able to detect telomerase in a large number of primary cell lines and primary human tissues. In contrast to normal human cells, cancer cells from tissue culture and those taken directly from tumors contain detectable telomerase activity. Counter, *et al.* (1994) *Proc. Natl. Acad. Sci. USA 91*:2900-2904; Kim, *et al., supra*. These findings suggest that targeting telomerase may be an effective cancer treatment. Harley, *et al.* (1994) *Cold Spring Harbor Lab Symposium on Quantitative Biology, 59*:307-315. However, to understand the regulation of telomerase in human diseases and disorders, it is essential to understand how telomerase functions.

Summary of the Invention

Described herein are truncated vertebrate (*e.g.*, mammalian and particularly human) telomerase and DNA oligonucleotides comprising truncated segments of the gene encoding telomerase RNA component, such as human telomerase RNA component, which are essential for telomerase activity (e.g., human telomerase activity) in cells and tissues. Also disclosed are DNA and RNA oligonucleotides sharing the biochemical and biological function of these essential oligonucleotides and differing only in alteration, substitution and/or deletion of one or more nucleotides (nt) which do not affect the activity of the enzyme. These oligonucleotides can be derived from other vertebrates, especially mammals. Oligonucleotides which hybridize to the above-described DNA or RNA sequences are also included

within the scope of this invention.

The truncated vertebrate telomerase of this invention is an essential oligonucleotide (RNA) and telomerase protein. The protein component can be comprised of more than one subunit. Preferably the RNA is encoded by DNA selected from nucleotides 44-204 of hTR as shown in Figure 6 (SEQ ID NO:1). Other essential oligonucleotides include sequences encompassing nucleotides 1-203, 1-273, 1-418, or DNA encompassing nucleotides 44-204 and sequential deoxyribonucleotides but shorter than nucleotides 1-445 (SEQ ID NO:2) of hTR as shown in Figure 6. Further provided is a 30 nucleotide sequence (nucleotides 170-199 of SEQ ID NO:1) which is required for functional RNA component activity.

Both the complete RNA and protein components of telomerase were thought to be necessary for telomerase activity and, thus, for maintenance of telomeric length in chromosomes. However, as described herein, truncated telomerase, in which the RNA component is shorter than the complete RNA component of human telomerase has been produced and shown to have enzymatic activity. The truncated human telomerase described herein can be produced by combining telomerase protein components with oligonucleotides prepared by recombinant methods, oligonucleotides which are isolated from sources in which they occur in nature or oligonucleotides which are synthetically produced. Similar types of truncated telomerases can be constructed by combining truncated oligonucleotides from other vertebrate telomerase RNA components with telomerase protein.

This invention also provides recombinant vertebrate telomerase in which the components are telomerase protein and the entire RNA component or a truncated RNA component, such as those encoded by nucleotides 44-204 of hTR as shown in Figure 6 (SEQ ID NO:1) or other essential

oligonucleotides including sequences encompassing nucleotides 1-203, 1-273, 1-418, or DNA encompassing nucleotides 44-204 and sequential deoxyribonucleotides but less than 1-445 of hTR as shown in Figure 6, or the 30 nucleotide sequence (nucleotides 170-199 of SEQ ID NO:1) which is required for functional RNA component activity. The telomerase protein can be synthesized, produced recombinantly or obtained from sources in which it occurs in nature.

The oligonucleotides of this invention can be used by themselves or combined with the protein of vertebrate telomerase for use in diagnostic or therapeutic methods and in assays for telomerase. Oligonucleotides that encompass the essential region of vertebrate telomerase are especially useful to block the function of telomerase by, for example, forming triple helices with DNA encoding RNA components, preventing transcription.

In another aspect of this invention, essential oligonucleotides may serve as probes or primers to detect the presence of telomerase in cells and tissues. Such probes or primers can be used diagnostically to determine the presence and amount of telomerase in cell, tissue or fluid samples obtained from an individual.

The oligonucleotides of this invention as well as the vertebrate telomerases described above can be used to treat disorders arising from the presence of normal or abnormal telomerase or to provide telomerase wherever it could be beneficial. Oligonucleotides in a sense or antisense orientation can prevent or inhibit telomerase activity by binding to essential regions of the RNA component or to telomerase protein. Sense or antisense sequences can be delivered with or without telomerase protein by methods of gene therapy (such as infection or transfection), as can plasmid or expression vectors encompassing recombinant DNA encoding vertebrate telomerase.

Pharmaceutical compounds consisting of oligonucleotides, telomerase, or truncated telomerase, alone or combined with a suitable carrier, diluent or salt are also included in this invention. These compounds can be therapeutically applied to stimulate or modify the effects of telomerase in order to treat conditions, disorders or diseases arising from the lack of or abnormal telomerase activity. Examples of such uses include initiation or restoration of telomerase activity to counteract senescence or to prevent immortalization, and prevention or inhibition of telomerase activity in immortalized cells such as tumor cells or parasites.

Another important feature of this invention is the use of the truncated or recombinant telomerase to screen for telomerase inhibitors which can be used to prevent telomerase expression or activity in cells and tissues. Telomerase activity of invading eukaryotic parasites or tumors can also be detected and quantified. Therefore, the present invention provides a diagnostic tool through which inhibitors of telomerase activity can be tested and developed, and by which diseases such as cancer, or infections, such as yeast or protozoan diseases, can be diagnosed.

Brief Description of the Drawings

Figure 1 shows that the reconstitution of human telomerase activity after MNase treatment is specific to hTR.

Figure 2 shows the activity of telomerase reconstituted with telomerase RNA mutations and assayed in the absence or presence of dATP.

Figure 3 represents a functional analysis of 5' and/or 3' terminal deletions of hTR.

Figure 4 represents a mutational analysis of hTR residues 170-199.

Figure 5 is a linear representation of full length hTR which includes the template region (white box) and positions of several restriction sites present in the gene encoding hTR.

Figure 6 is the nucleotide sequence (SEQ ID NO:1) of the gene encoding the human RNA component of telomerase with the template boxed. The cleavage sites for several restriction endonucleases are marked.

Figure 7 is the hTR sequence used for several hTR reconstitution experiments with the template and cleavage sites for restriction endonucleases marked.

Detailed Description of the Invention

This invention provides isolated DNA encoding portions of the RNA component of human telomerase (hTR) that are essential to produce a biologically active human telomerase enzyme. The term "hTR" is used interchangeably for the RNA component or the gene encoding the RNA component. Those of skill in the art will recognize which type of nucleic acid is intended where appropriate in this description. This invention also provides truncated human telomerase RNA which, in combination with telomerase protein, produces biologically active human telomerase (i.e., one which catalyzes the addition of deoxyribonucleotides to the telomeres of chromosomes, thereby elongating the telomeres of these chromosomes). The essential oligonucleotides described herein are substantially shorter (comprise fewer nucleotides) than the endogenous human RNA component. As used herein, the term "essential" oligonucleotides refers to oligonucleotides which, when coupled with the human telomerase protein, form biologically active telomerase and without which biologically active telomerase is not produced. Both RNA that is essential to functional telomerase and DNA encoding RNA that is essential are referred to as "essential oligonucleotides" (essential DNA,

essential RNA). Essential DNA of this invention includes isolated DNA sequences of hTR selected from the group consisting of:

a) nucleotides 44-204 of hTR; b) nucleotides 1-203, 1-273, or 1-418 of hTR; and c) DNA encompassing nucleotides 44-204 and sequential deoxyribonucleotides but shorter than 1-445 of hTR. It further includes nucleotides 170-199 of hTR which are essential for telomerase activity although additional nucleotides are required to provide a biologically active RNA component.

This invention encompasses isolated DNAs whose sequences are provided (Figures 5 and 6) and other DNAs which encode the same RNA sequences. This invention further provides DNA which hybridizes to the essential DNA described above, especially under stringent conditions such as those described in Ausubel, *et al.* (1995) *Current Protocols in Molecular Biology - A Laboratory Manual*, Chapter 6, John Wiley & Sons, NY, and DNA sequences which, but for the degeneracy of the genetic code, would hybridize to the essential DNA described above.

Applicants have discovered that the entire RNA sequence of the RNA component of human telomerase as shown in Figure 6 is not required for telomerase activity. In fact, only certain portions of the RNA component are essential to produce an active telomerase (e.g., by combining with human telomerase protein). These encompass the template of the RNA component and a minimum number (160) of additional ribonucleotides upstream and downstream along the molecule (See encoding DNA molecule in Figure 6, nucleotides 44-204).

This invention also provides, for the first time, functional vertebrate telomerase, produced with the complete nucleotide sequence of the RNA component or with the essential oligonucleotides of the RNA component (sequences ranging from 160 to 445 nucleotides in length)

which have been delineated by the Applicants.

It should be noted that truncated vertebrate telomerases (constructed with an RNA component comprising fewer ribonucleotides than the endogenous RNA component of the same species), for the first time, provide telomerases modified by deletion of nonessential ribonucleotides and permit the production of telomerase variants which retain telomerase binding activity. These variants are useful in the treatment of conditions such as cell senescence (ageing) and in diseases as anti-tumor drugs.

The following generally describes the reconstitution of recombinant human telomerase and the discovery of the essential oligonucleotides for telomerase activity. More specific methodology can be found in the examples.

Reconstitution of human telomerase activity after MNase treatment

To determine whether human telomerase activity could be reconstituted from isolated protein and RNA components, partially purified human telomerase extracts were treated with MNase to remove endogenous telomerase RNA. Telomerase activity was followed by a modification of the TRAP assay (Kim *et al.*, *supra*) (see Exemplification). After nuclease digestion, which abolished endogenous telomerase activity, activity was restored by incubating MNase-treated telomerase with EDTA and an *in vitro* transcribed hTR transcript (hTR1-557), followed by the addition of Mg^{2+} (Figure 1). The hTR1-557 was transcribed from plasmid pGEM33 digested with *Eco*RV. The hTR1-557 contains the entire hTR (445 nucleotides (nt)) plus downstream sequences (112 nt). Vector sequences 5′ and 3′ to hTR genomic sequence were also transcribed so that the total length of the transcript is 630 nt. When no RNA was added, no telomerase activity was restored. To test for linearity in the telomerase reaction, two different concentrations of

extract (6 μl and 12μl) were used and to test for linearity in the reconstitution, two different concentrations of RNA were added (0.4 μg and 0.8 μg). The amount of reconstituted activity increased with the increased level of both the extract and hTR indicating that reconstitution was dependent on the added RNA (Figure 1).

Reconstitution is specific to human telomerase RNA

To determine whether reconstituted activity was specific to hTR, several nonspecific RNAs were tested in place of hTR in the reconstitution assay. The RNAs tested were *E. coli* 5S, *E. coli* 16S and 23S RNAs, and *Tetrahymena* telomerase RNA (Figure 1). No activity was seen when these RNAs were added instead of hTR in the reconstitution assay. Also, no T_2G_4 repeats were generated by adding *Tetrahymena* telomerase RNA to MNase-treated human extract, using the C-strand primer C_4A_2 to detect the presence of amplified elongation products (see below and Exemplification). Activity was also not reconstituted using the mouse RNase P RNA, the antisense strand of hTR and the mouse telomerase RNA (mTR) (Blasco *et al.*, (1995) *Science 269*:1267-1270).

To determine whether the signal in the TRAP assay was dependent on human telomerase extract and not due to a reaction involving amplification of the added hTR alone, reconstitution was performed in the absence of human telomerase extract. No amplified products were detected under these conditions indicating reconstitution is dependent on protein components. To further test the specificity of the reconstituted telomerase activity, experiments were performed using telomerase RNAs with mutations in the template region. Both *in vivo* and *in vitro* experiments with *Tetrahymena* telomerase showed that altering the template region of the telomerase RNA results in reprogramming the sequence that telomerase synthesizes (Yu *et al.* (1990) *Nature 344*:126-132; Autexier and Greider

(1994) *Genes & Dev.* *8*:563-575). Similarly when the genes encoding the human or mouse telomerase RNAs were mutated in the template region and the genes transfected into cultured cells, telomerase activity was isolated which synthesized the expected mutant telomere repeats (Blasco *et al.*, 1995 *Science* *269*:1267-1270; Feng, *et al.* (1995) *Science* *269*:1236-1241.

Analogous hTR mutants were used in the *in vitro* reconstitution experiments. In the plasmid pGEM34, the sequence encoding the template region of hTR was changed from CTAACCCTA to C<u>A</u>AACCC<u>A</u>A (encoding hTR-C_3A_3) and in pGEM36 to C<u>C</u>AACCC<u>C</u>A (encoding hTR-C_4A_2), which should specify TTGGGG and TTTGGG repeats, respectively. The RNA transcribed from these plasmids, hTR-C_3A_3 and hTR-C_4A_2, like hTR1-557, contain sequences downstream of hTR and vector sequences (see Exemplification). To assay the products of the mutant telomerases, a two step amplification protocol was used as described (Feng, *et al.* (1995) *Science* *269*:1236-1241). In the first step, dATP was omitted from the initial telomerase reaction. Under these conditions wild type (naturally-occurring) telomerase will not generate elongation products, however if the mutant RNAs (hTR-C_3A_3 and hTR-C_4A_2) are functional, they should generate telomerase products. For the PCR amplification step, dATP was added and the C-strand primer corresponding to the appropriate mutant was used for PCR amplification (see Exemplification).

Amplified elongation products were detectable in the absence of dATP for the mutants but not for the wild type RNA added in reconstitution, indicating the requirement and specificity of hTR in the *in vitro* reconstitution of human telomerase activity (Figure 2). No long elongation products are generated with the addition of hTR-C_4A_2, as was seen *in vivo* (Feng, *et al.*, *supra*). Telomerase reconstitution was also assayed with hTR1-557 containing a

17 base insertion at residue 176 (hTR+17) (Feng, *et al.*, *supra*). Only very weak elongation products were detectable with this mutant. In other experiments this RNA failed to give significant levels of reconstitution. These results are consistent with results from the *in vivo* reconstitution of mutant RNAs in human cells, where this RNA was also not functional (Feng, *et al.*, *supra*). The presence of the 17 base insertion at nucleotide position 176 appears to inhibit the function of the human telomerase RNA.

Identification of a 160 nucleotide minimal functional region of hTR between residues 44-204

The 450 nucleotide human telomerase RNA is much larger than the *Tetrahymena* (160 nt) and other ciliate telomerase RNAs (147-209 nt), however it is significantly smaller than the yeast telomerase RNA (1300 nt) (Greider and Blackburn (1989) *Nature 337*:331-337; Lingner *et al.* (1994) *Genes & Dev. 8*:1984-1998; Singer and Gottschling (1994) *Science 266*:404-409; Feng, *et al.*, *supra*; McEachern and Blackburn (1995) *Nature 376*:403-409; McCormick-Graham and Romero (1996) *Mol. Cell. Biol. 16*:1871-1879; Zaug *et al.* (1996) *Nucleic Acids Res. 24*:532-533). To determine the essential functional regions of the hTR and whether the entire RNA sequence is required, telomerase activity was reconstituted with RNAs deleted at the 5' and/or 3' ends (Figures 3 and 5). For a more accurate analysis of hTR, a plasmid encoding only hTR was constructed that will generate an RNA without downstream genomic sequences or vector sequences (upstream or downstream) (phTR+1; see Exemplification). To generate deletions in the 3' end of hTR, RNA was transcribed from phTR+1 that had been cut with specific restriction endonucleases at various positions within the coding region of hTR. Full length wild-type hTR is denoted as hTR1-445. The numbers refer to the position of residues within the full length hTR. Each RNA, with the enzyme used

to cut the plasmid (in brackets) is denoted as hTR1-445 (*Fsp*I), hTR1-418 (*Apa*LI), hTR1-273(*Bsp*E1), hTR1-203 (*Sma*I), hTR1-182 (*Pvu*II), hTR1-169 (*Bbv*I) and hTR1-159 (*Xba*I). To generate the RNAs deleted at both the 5′ and 3′ ends (hTR44-170, hTR44-184 and hTR44-204), RNA was transcribed using PCR fragments generated using primers that anneal at the respective positions of the gene encoding hTR (see Exemplification). Different amounts (1.25, 2.5 and 5 pmol) of each hTR deleted at the 3′ end were added in the reconstitution reaction. Activity increased with increasing amounts of RNA.

Significant levels of activity were restored with 2.5 pmol of hTRs beginning at postion +1 and extending 445, 418, 273 and 203 nt in length. Quantitation of the amplified elongation products (see Exemplification) indicated that the addition of hTR1-182 and hTR1-169 restored little activity (1-3% compared to the addition of full length hTR1-445) and activity was undetectable with a hTR 159 nt in length (hTR1-159). The relative activities of the 3′ deletions of hTR are summarized in Figure 5. This deletion analysis showed that 242 residues at the 3′ end are not essential for telomerase activity. These results also suggest that a region of hTR, approximately 44 residues in length, between positions 159 and 203 is important for hTR function. RNAs were then tested which were truncated at both the 5′ and 3′ ends that contained residue 44 through to either residue 184 or 204. Both of these RNAs were active in reconstitution, although they had reduced activity compared to the addition of full length hTR. An hTR truncation starting at position 44 and ending at residue 170 (hTR44-170) was not active in reconstitution, indicating that a region of hTR, approximately 33 residues in length, between 170 and 203 is important for hTR function. The ability of RNAs containing only residues 44-184 and 44-204 to reconstitute activity

suggest that the 44 residues preceding the template are not essential for activity.

A 30 nucleotide region of hTR spanning residues 170-199 is essential for activity

To more clearly define the role of residues 170 to 203 of hTR in telomerase function, substitutions were made spanning residues 170 to 179 (hTR170*), 180 to 189 (hTR180*) and 190 to 199 (hTR190*). Sequences in phTR+1 at positions 170-179 (5'-CAAAAAATGT-3') were replaced by 5'-GTTTTTTACA-3', at positions 180-189 (5'-CAGCTGCTGG-3') by 5'-GTCGACGACC-3' and at positions 190-199 (5'-CCCGTTCGCC-3') by 5'-GGGCAAGCGG-3'. Different amounts of these hTRs (1.25, 2.5 and 5 pmol) were tested in reconstitution and there was an increase in activity with increasing amounts of RNA added. Levels of telomerase activity reconstituted with 2.5 pmol of these RNAs were compared to that reconstituted with three 3' deletions (hTR1-159, hTR1-169 and hTR1-182) or hTR1-445 (Figures 4 and 5). Reconstitution with either hTR170*, hTR180*, hTR190* restored little activity, comparable to the activity restored by hTR1-169 and hTR1-182 (less than 8% of activity restored by hTR1-445). These results suggest that either the sequences or potential secondary structures in the 30 nucleotide region between 170 and 199 are essential for activity. In addition this region contains the site of the 17 nucleotide insertion that disrupted the ability of hTR to function *in vitro* and *in vivo*. Thus these mutants define an essential functional region of the hTR.

Thus, this invention delineates the essential oligonucleotides necessary to reconstitute a functional human RNA component. The findings described herein are summarized as follows. Human telomerase activity was restored to MNase-treated partially purified human telomerase by the addition of EDTA and *in vitro* transcribed

human telomerase RNA, as previously described for *Tetrahymena* telomerase (Autexier and Greider (1994) *Genes & Dev. 8*:563-575). The levels of reconstituted activity compared to native activity varied, but were always lower (less than 10%), as was the case for levels of reconstituted *Tetrahymena* telomerase, suggesting that the added hTR may not be completely functional compared to endogenous telomerase RNA. The transcribed RNA may lack some modifications or assume incorrect conformations which prevent it from forming a functional RNP (Autexier and Greider (1994) *Genes & Dev. 8*:563-575). The extra sequences downstream of hTR and the transcribed vector sequences did not inhibit the ability of hTR1-557 to reconstitute telomerase activity, compared to full length hTR1-445.

The inability to restore human telomerase activity with the mouse telomerase RNA (mTR), even though human and mouse telomerase both catalyze the addition of T_2AG_3 repeats, indicates a requirement for species-specific telomerase protein-RNA interactions. In *Tetrahymena*, the telomerase enzyme is about 250 kDa and consists of two proteins, of 80 and 95 kDa (Collins *et al.* (1995) *Cell 81*:677-686). The predicted sizes of the human (750 kDa) and mouse telomerase (>1000 kDa) enzymes differ from each other, and are larger than the *Tetrahymena* telomerase enzyme (Greider *et al.* (1996) *In:* M. DePamphlis, ed., *DNA replication in eukaryotic cells*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., *in press*). The mouse telomerase enzyme may consist of more or larger protein components than the human enzyme and consequently, at least *in vitro*, mTR may be unable to form a functional complex with the human telomerase proteins.

Altering the template region of hTR changed the sequence of the elongation products generated by reconstituted telomerase, as seen with *Tetrahymena* ,*in vivo*

and *in vitro*, (Yu *et al.* (1990) *Nature 344*:126-132; Autexier and Greider (1994) *Genes & Dev. 8*:563-575) and human and mouse (Blasco *et al.*, 1995, *supra*; Feng, *et al.* (1995) *Science 269:1236-1241*, *in vivo*, confirming the requirement and specificity of hTR in the *in vitro* reconstitution of human telomerase activity. The specificity and fidelity of reconstituted activity suggests that the reconstitution assay will be a useful biochemical tool for dissecting native human telomerase function, as it has been for *Tetrahymena* (Autexier and Greider, (1995) *Genes & Dev. 15*:2227-2239).

Greater than half of the 445 nt hTR is not absolutely required for telomerase activity *in vitro*, including residues 5′ to the template. These studies define a minimal functional region of hTR, approximately 159-203 nt length. This minimal function region is similar in size to the full length telomerase RNAs from ciliates, which range in size from 147-208 nt (Greider, *et al.* (1996); McCormick-Graham and Romero, *supra*). However, reduced activity of telomerase reconstituted with the deleted hTRs which are still functional, compared to activity reconstituted with full length hTR, suggests that the deleted regions may contain sequences or potential secondary structures important for binding of telomerase protein components, for assembly and for overall structure and function of the telomerase complex. It is also possible that the remainder of the RNA plays some role *in vivo*, perhaps by binding proteins important in the regulation of telomerase. This deletional analysis of hTR, and the size of the telomerase RNAs in *S. cerevisiae* and *K. lactis* (1300 nt) suggest that the entire telomerase RNA in these organisms may not be needed for function. In yeast, the U2 snRNA is 1175 nt long compared to in most other organisms where it is about 190 nt long (Ares, (1986) *Cell* 47:49-59). Internal deletions which reduce the length of

the yeast U2 snRNA to that of other U2 snRNAs are still active in splicing and yeast with the deleted U2 snRNA have normal growth rates (Igel and Ares (1988) *Nature 334*:450-453; Shuster and Guthrie (1988) *Cell 55*:41-48). The yeast U1 snRNA is also larger (568 nt) than in metazoans where it is 165 nt. Yeast cells carrying a deletion of 316 internal residues allows wild-type growth (Siliciano *et al.* (1991) *Nucleic Acids Res. 19*:6367-6372). Deletional analysis of hTR (*in vivo*) and yeast telomerase RNA will be required to further elucidate the role of the extra residues in these longer telomerase RNAs. It is not clear, for hTR, mTR, or the yeast telomerase RNAs, if there is a minimal core conserved secondary structure for all of these RNAs which is essential, like with RNase P (Waugh *et al.* (1989) *Science 244*:1569-1571).

Since deleted forms of hTR were active in reconstitution, theoretically, small pieces of endogenous hTR could remain following MNase digestion which could intermolecularly complement the synthetic hTR forms. Northern analysis of MNase-treated extract, however, indicate that hTR is digested into pieces smaller than 50 nt. Further, titrations of the synthetic hTR deletions showed that the amount of reconstituted activity increased with increasing amounts of hTR, demonstrating that reconstitution was dependent on the added RNA and not endogenous RNA. Since the amount of RNA added was approximately 1000 fold over that orginally present in the extract, it is clear that the reconstituted activity was due to the addition of synthetic RNA and not to the complementation of synthetic RNA by the un-degraded portions of the RNA.

In *Tetrahymena* and other ciliate telomerase RNAs, there is a conserved region upstream of the template, which plays a role in determining the 5' boundary of the template, and the sequence synthesized by telomerase *in*

vitro (Romero and Blackburn (1991) *Cell* 67:343-353; Autexier and Greider, 1995, *supra*). This sequence is absent in hTR, mTR and yeast telomerase RNAs (Singer and Gottschling (1994) *Science* 266:404-409; Blasco *et al.*, 1995, *supra*; Feng, *et al.*, *supra*; McEachern and Blackburn (1995) *Nature* 376:403-409. The absence of this sequence and the ability of hTR44-204, which lacks sequences 5′ to the template, to reconstitute telomerase activity, suggest that human, mouse and yeast may use a different mechanism to regulate the 5′ template boundary. Most of the telomerase RNAs contain templates which are located approximately 50 nt from the 5′ end, except for yeast where the template is more centrally located (Singer and Gottschling, *supra*; McEachern and Blackburn, *supra*). In the *Tetrahymena* telomerase RNA, which is only 159 nt in length, deletions of as little as 19 residues from the 5′ end abolish activity indicating that residues 5′ to the template are essential. In hTR, residues 5′ to the template are not essential *in vitro*. However, the 5′ end may play some role, *in vivo*, perhaps by maintaining a correct RNA structure for proteins to interact with other sequences or structures of hTR.

Mutagenesis of sequences spanning residues 170-179, 180-189 or 190-199 of hTR almost completely abolished the ability of hTR to function in reconstitution, suggesting that these sequences are functionally and/or structurally important, perhaps by binding telomerase or other proteins. Low levels of activity were detectable when telomerase is reconstituted with hTR170*, hTR180* or hTR190*, suggesting that proteins may still bind weakly to the other 20 nt which are not mutated in each case. Similarly, insertion of 17 nt at position 176 also dramatically decreased activity, providing further evidence that the structure or sequence of this 30 nt region is important.

The ability to reconstitute human telomerase activity

using inactive protein and mutant hTR has allowed a functional dissection of telomerase. With the definition of a minimal functional region of hTR, the role of specific sequences in this region and their importance for function can now be tested directly using the reconstitution assay. With the identification of human telomerase protein components a thorough understanding of human telomerase protein-interactions and of the mechanism of human telomerase action will be possible.

In one aspect, this invention provides nucleic acid hybridization probes or primers which hybridize to a sample nucleotide sequence, its complement or to a fragment of either of these. Thus a method of determining the presence of telomerase in a cellular sample obtained from an individual is available. Methods of detecting telomerase with an essential oligonucleotide (DNA or RNA) in a cell, tissue, or fluid sample include the steps of: preparing the sample so that the essential oligonucleotide will hybridize to telomerase in the sample; combining or contacting the sample with the DNA or RNA under conditions under which hybridization of complementary nucleic acids occurs; and detecting hybridization wherein if hybridization occurs, telomerase is present in the sample. Further assays can be carried out to confirm whether telomerase is active. An additional step can also be taken to measure the amount of hybridization to determine the amount of telomerase in the sample. These essential oligonucleotide probes can be detectably labeled (for example with radioactive or fluorescent materials, or with biotin or avidin) by methods known to those of skill in the art.

In the same manner, primers which are all or a portion of essential oligonucleotides can be used to initiate DNA synthesis for amplification or diagnostic procedures. If a primer is a portion of an essential oligonucleotide, it

must be of sufficient length to hybridize to DNA and remain hybridized under the conditions used, although the nucleotides may not be identical in sequence. In general, a primer will be at least 12 nucleotides and can be up to 100 nucleotides in length. Preferably primers will be 18 to 30 in length. The primers may be labeled before hybridization so that detection of labeled hybridized material correlates with the presence and/or amount telomerase in a sample taken from an individual. These methods can be carried out following amplification of the RNA component for early detection of diseases, such as cancers or parasites, where only a few cells may be present in the sample. They also relate to procedures wherein recombinant telomerase is synthesized (with a whole or truncated RNA component) and an active telomerase enzyme produced.

Knowing what sequences are essential for activity could also make it possible to determine whether alteration of the endogenous RNA component has occurred in a portion of the molecule necessary for activity. Alterations, substitutions, or deletions of nucleotides, or other abnormalities in essential regions may inhibit or inactivate the enzyme so that telomeres of chromosomes are not lengthened.

Another aspect of this invention relates to the use of the isolated DNA sequences in antisense therapy to block telomerase activity. Antisense therapy refers to administration of or *in situ* generation of oligonucleotides or their derivatives which specifically hybridize with the endogenous telomerase RNA component and/or which hybridize with genomic DNA encoding the RNA component so as to inhibit expression of that enzyme, e.g., by inhibiting transcription and/or translation.

This invention also relates to antisense constructs that can be delivered, for example, in an expression vector

that, when transcribed in the cell, produces RNA which is complementary to at least the essential portions of the telomerase RNA component. Expression vectors, such as plasmids, are capable of directing the expression of genes to which they are operatively linked. Alternatively, the antisense construct is an oligonucleotide which is generated *ex vivo* and which, when introduced into the cell, causes inhibition of expression by hybridizing with the telomerase RNA component or by hybridizing with genomic sequences encoding the RNA component, thus preventing telomerase from serving as a template for telomeric DNA synthesis. Such oligonucleotides are preferably modified oligonucleotides which are resistant to endogenous nucleases and therefore stable *in vivo*. General approaches to constructing oligomers useful in antisense therapy have been described, for example, in Inouye, U.S. Patent No. 5,272,065, incorporated herein by reference, and reviewed by Stein, *et al.* (1988) *Cancer Res. 48*: 2659-2668.

There are other methods by which the endogenous telomerase can be inactivated. For example, insertion of gene sequences into target tissues in a sense orientation can be used to produce RNAs that bind to essential telomerase protein, thereby inactivating the enzyme. For example, a DNA construct encoding an RNA essential oligonucleotide can be linked to a strong promoter to express excess sense strands at a high level which competitively inhibit the specific binding of an essential protein. Other sense sequences may be constructed that will bind to the DNA essential oligonucleotides to form a triple helix and prevent transcription. Knowledge of the essential portions of the RNA component increases the likelihood of success for these endeavors because these constructs contain nucleic acid sequences that will bind.

Oligonucleotides that bind to the RNA component of telomerase may also be combined with ribozyme sequences to

produce molecules that not only bind but specifically cleave the RNA component, thus inactivating telomerase.

Both RNA and protein components are involved in telomeric primer recognition and binding by telomerase (Collins and Greider (1993) *Genes & Dev.* 7:1364-1376). The ability to reconstitute human telomerase activity from partial RNA component sequences and the telomerase protein not only facilitates the structural and functional dissection of this ribonucleoprotein, it allows the production of fundamental synthesized enzymes with multiple applications.

Truncated or recombinant human telomerase in all of the disclosed forms can be used to design drugs or produce pharmaceutical compositions for treating disorders in which telomerase activity would be beneficial. Functional telomerase molecules can be delivered to cells to stimulate telomerase activity in cells normally lacking detectable telomerase or in cells which are abnormal because telomerase activity is present. Telomerase can be used to extend replicative cell life span and deter cell senescence and possible subsequent immortalization of cells.

Accordingly, the modified oligomers and telomerase enzymes of the invention are useful in therapeutic, diagnostic and research contexts. Recombinant telomerase can be especially useful in therapy where it is important to slow the loss of telomere sequences (i.e., preventing senescence of cells).

Pharmaceutical compositions containing the telomerases of this invention can be used to treat conditions such as those described above. Additionally, the telomerase molecules can be used in screening other agents, for example, in binding assays, to identify compounds which inhibit or stimulate the activity of telomerase *in vitro* or *in vivo*.

Pharmaceutical compositions containing the telomerases

or essential oligonucleotides of this invention may also contain pharmaceutically acceptable carriers, diluents, fillers, salts, buffers, stabilizers and/or other materials well known in the art. The term "pharmaceutically acceptable" means a non-toxic material that does not interfere with the effectiveness of the biological activity of the active ingredients(s). The characteristics of the carrier or other material will depend on the route of administration which can be carried out in a variety of conventional ways. The amount of the active ingredient(s) in the pharmaceutical composition of this invention will depend upon the nature and severity of the condition being treated, and may depend on the nature of any prior treatments which the individual has undergone. In any event, such methods require the administration of a therapeutically effective amount of the active ingredient(s) which is at least the minimum amount necessary to effect a beneficial change in the condition being treated.

It will be appreciated that the actual preferred amounts of active compound in a specific case will vary according to the specific compound being utilized, the particular compositions formulated, the mode of application, the particular situs of application, and the individual being treated. Dosages for a given recipient will be determined on the basis of individual characteristics, such as body size, weight, age and the type and severity of the condition being treated.

It should be noted that the formulations described herein may be used for veterinary as well as human applications and that the term "individual" or "host" should not be construed in a limiting manner.

Primary cells express little or no telomerase activity, but following immortalization, cancer cells reactivate telomerase and maintain telomere length. In

fact, telomerase activity has been demonstrated in human ovarian carcinoma cells, but not in normal cervical endothelial cells. Counter, *et al.* (1994) *Proc. Natl. Acad. Sci. USA 91*:2900-2904. Telomere shortening before crisis may be lethal, but those cells that can reactivate telomerase maintain telomere length and survive crisis. This model suggests that if telomerase is required for the growth of immortalized cells, telomerase inhibitors may be excellent anti-cancer drugs.

This invention provides a method by which cancers may be diagnosed prior to or during clinical manifestation of symptoms by means of detecting telomerase activity in somatic cells that normally do not express telomerase. Telomerase RNA expression in a sample of somatic cells or tissue can be detected using the DNA or RNA probes described herein; this is indicative of expression of telomerase which, in turn, is an indication of immortal cancer cells since most somatic cells do not normally produce telomerase. Detection of hybridization in tissues that normally lack telomerase is an indication of a predisposition to cellular immortalization or cancer, or to the presence of cancer or immortal cells.

By example, such methods of detecting the presence of immortal cells or a predisposition to immortalization in a eukaryotic cell, tissue or fluid sample can include: obtaining a cell, tissue or fluid sample; and using the essential oligonucleotides to determine the presence of telomerase in the sample (for example, by hybridization with a labeled probe), wherein if the sample demonstrates the presence of telomerase, immortal cells or the predisposition to immortalization is present. The same method may be used to detect a predisposition to cancer or the presence of cancer cells or tissue.

Alternatively, the recombinant telomerase can be used to produce polyclonal or monoclonal antibodies to the

telomerase protein. These antibodies allow detection of telomerase *in vivo* or *in vitro* at minute levels and can serve to indicate the presence of abnormal telomerase activity due to tumor cell growth or other conditions such as parasitism by foreign eukaryotic organisms (i.e., yeasts, protozoa), and the like. Because antibodies can accurately detect small amounts of antigen, early diagnosis of these disorders is possible.

The present invention also provides a means for developing drugs and pharmaceutical compounds that destroy or otherwise inactivate or interfere with the activity of telomerase. Thus, the truncated or recombinant telomerase, or the essential oligonucleotides of this invention can be used to screen for potential new drugs and pharmaceutical compounds effective as anti-cancer and anti-microbial agents, as described below. Further, since additional telomerase activity may have an anti-aging effect and result in restoration of cells by stabilizing telomere length, compounds which stimulate or trigger telomerase activity can be identified.

For example a method for screening agents which inhibit, prevent, or stimulate telomerase activity can comprise the steps of: contacting the potential agent with truncated or recombinant telomerase under conditions wherein telomerase is active; and determining whether the activity of telomerase is decreased or increased; whereby if the telomerase activity is decreased, the agent is identified as a telomerase inhibitor and, if the telomerase activity is increased, the agent is identified as a telomerase stimulator.

The telomerase protein can also be combined with the RNA component of telomerase to produce a functional recombinant telomerase molecule which can be delivered to cells by conventional methods. Alternatively, DNA encoding a telomerase molecule can be introduced into target cells

by recombinant DNA methods and transformation technology. The incorporation of extra copies of functional telomerase molecules may extend the replicative life span of the host cell by stabilizing telomere length. Thus, this invention includes methods for targeted gene therapy in individuals.

Another application of this invention is the detection of eukaryotic disease-causing organisms in somatic cells and tissues of vertebrates and treatment of the resulting disease. There are many fungi, protozoa, and even algae that invade the cells and tissues of vertebrates and are the cause of various diseases. Examples of such diseases include, but are not limited to, aspergillosis, histoplasmosis, candidiasis, paracoccidioidomycosis, malaria, trichinosis, filariasis, trypanosomiasis (sleeping sickness), schistosomiasis, toxoplasmosis, and leishmaniasis. These organisms probably require telomerase and express this enzyme as they multiply inside host cells which do not normally produce telomerase. The above-described methods to detect telomerase can be used to develop early detection and diagnosis procedures for these eukaryotic microbial parasites.

An example of such a method to detect a disease caused by a eukaryotic microbial organism in a sample of cells from an individual may comprise the steps of: obtaining a sample of cells from the individual; and determining if microbial telomerase is present in the sample; wherein if the sample demonstrates telomerase of a eukaryotic microbe, a disease caused by a eukaryotic microbial organism is present. If telomerase is normally present in the cells of the individual, e.g., germline cells, the microbial telomerase can be distinguished by determining if hybridization occurs with a probe specific for non-human telomerase.

Furthermore, since most mammalian somatic cells do not require telomerase, the use of inhibitors of and

antibiotics against telomerase will provide a method of treatment for such diseases that is nontoxic or exhibits little toxicity to the host. For example, most of the drugs used to treat diseases caused by *Trypanosoma* species can cause serious side effects and even death. Use of antisense RNA to the RNA of *Trypanosoma* sp. telomerase or drugs against telomerase may inhibit telomerase and thus prevent the multiplication of species of this parasite in an individual without affecting the host's somatic cells and tissues. Included among these pharmaceuticals are nucleic acids complementary to essential oligonucleotides (antisense) that inhibit the expression of telomerase.

In a further aspect, the present invention provides a process for producing a recombinant product comprising: producing an expression vector which includes DNA which encodes a telomerase molecule; transfecting or infecting a host cell with the vector; and culturing the transfected or infected cell line to produce the encoded telomerase molecule (recombinant telomerase). The standard techniques of molecular biology can be used to prepare DNA sequences coding for the RNA and protein components of telomerase, and for construction of vectors with appropriate promoters for enzyme expression in a host cell. Suitable host cell/vector systems, transfection or infection methods and culture methods are well known in the art. These systems may also be used to produce antibodies to telomerase.

It will also be appreciated that the methods described above may be used to produce transgenic cells, tissues, and organisms for use in investigating the role of telomerase in eukaryotic organisms, and for therapeutic purposes.

The present invention will now be illustrated by the following examples, which are not intended to be limiting in any way.

EXEMPLIFICATION

Example 1

Preparation of human telomerase extracts

Fractions used for reconstitution were prepared in the following manner: S100 cytoplasmic extract was prepared from 293 cells as previously described (Counter *et al.* (1992) *EMBO J. 11*:1921-1929). A zero to 40% ammonium sulfate cut was made from this fraction, dialyzed to remove the ammonium sulfate and applied to a Toyopearl Q column equilibrated with buffer A containing 0.1 NaCl (A+0.1 NaCl buffer: 20 mM HEPES pH 7.9, 1mM DTT, 1 mM EGTA, 1 mM $MgCl_2$, 10% glycerol, 0.1 M NaCl). The column was washed with buffer A containing 0.18 M NaCl, and telomerase activity was eluted with buffer A containing 0.3 M NaCl. The active fractions were pooled and concentrated with 50% ammonium sulfate and applied to a Toyopearl HW-65F column equilibrated in buffer A containing 0.1 M NaCl and eluted with the same buffer. Active fractions (1.7 mg/ml total protein) were pooled and used in reconstitution.

Example 2

Telomerase elongation activity assay

Activity was assayed using a combination of the conventional telomerase assay (Counter *et al.* (1992) *EMBO J. 11*:1921-1929) and the TRAP assay (Kim, *et al.*, *supra*). The conventional conditions were: one hour incubation at 30°C in 1 X telomerase buffer (50 mM Tris-HCl pH 8.3, 1 mM DTT, 1 mM spermidine, 1mM $MgCl_2$), 2mM dATP, 2mM dTTP, 10 μM dGTP and 40 pmol M2 oligo (5'-AATCCGTCGAGCAGAGTT-3'). Different volumes of extract were assayed as indicated in the figures. Unless otherwise indicated, 12 μl was used (final 20 μl) and mixed 1:1 with the reaction mixture. 10 μl of the 40 μl telomerase reaction was then added to a 50 μl final volume PCR reaction: 1 X TRAP buffer (20 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 63 mM KCl, 1mM EGTA, 0.005% Tween-20, 0.1 mg/ml BSA), 50 μM dNTPs, 20 pmol M2 primer,

20 pmol of the appropriate C-strand primer, 0.13 μM [α-32P]dGTP (0.5 μl of 800 Ci/mmole; NEN) and 2U Taq polymerase (Perkin-Elmer). The C-strand primers, C_3TA_2 primer, C_3A_3 primer, or C_4A_2 primer were used to detect the corresponding G-rich telomerase elongation products T_2AG_3, T_3A_3 and T_2G_4. These primers are modified versions of the Cx primer (Kim *et al.*, *supra*) and contain three repeats of the appropriate telomeric sequence plus some additional sequences at the 5' end (Trap-eze™ kit, Oncor, Inc., 209 Perry Parkway, Gaithersberg, MD 20877, www.oncorinc.com/home). After amplification for 18 cycles at 30 sec 94°C, 30 sec 60°C and 30 sec 72°C, 7.5 μl of the reaction was mixed 50/50 with formamide containing xylene cyanol and bromophenol blue. Products were resolved on a 12%, 7M urea denaturing gel in 0.6 X TBE electrophoresed at 35 W until xylene cyanol was 5 cm from the bottom. Gels were dried and then exposed to Fuji PhosphorImager screens overnight and then to film (XAR5) for the times indicated in the figures. Products were quantitated by comparing the signal intensity in each lane using a BAS2000 PhosphorImager.

Example 3

MNase treatment and reconstitution conditions

Reconstitution conditions were modified from those for reconstitution of *Tetrahymena* telomerase (Autexier and Greider (1994) *Genes & Dev.* *8*:563-575). Twelfe μl of human telomerase fractions (in 1 mM EGTA) were treated for 10-15 min at 30°C with 1.9-2.1 mM $CaCl_2$ and 1.0-1.15 Unit of micrococcal nuclease (MNase) (Pharmacia) per μl of extract. The MNase was inactivated by the addition of 1.5 mM EGTA. The extract was then incubated with *in vitro* transcribed RNA and 5 mM EDTA for 5 min at 37°C. 8mM $MgCl_2$ was added prior to assaying for telomerase activity. Mock-treated telomerase consists of telomerase treated as described

above, with the addition of EGTA prior to MNase.

In Figure 1, telomerase assays were performed with telomerase pretreated as indicated. In lanes 1-12, MNase-treated telomerase was reconstituted: without addition of RNA (lanes 1 and 2); with 0.4 μg hTR1-557 (lanes 3 and 4); or, with 0.8 μg hTR1-557 (lanes 5 and 6); with 8 μg *E. coli* 5S rRNA (lanes 7 and 8); with 1.1 μg 16 and 23S rRNA (lanes 9 and 10); or with 0.8 μg *Tetrahymena* telomerase RNA (lanes 11 and 12). Either 6 or 12 μl of extract was used in the elongation assay, prior to amplification of elongation products, as indicated. 0.4 and 0.8 μg of hTR is equivalent to approximately 2 and 4 pmoles, respectively. The gel was exposed 18 hours on film.

Example 4

Construction of phTR+1, phTR170, phTR180 and phTR190

A 480 bp fragment containing the T7 promoter and positions +1 to 445 of the gene encoding hTR was generated by PCR from the cloned hTR gene (Feng, *et al.*, *supra*), digested with *Hind*III and *Bam*HI, and cloned into pUC119 digested with the same enzymes. The template used in PCR was a 794 bp *Eco*RI-*Fsp*I fragment from pGRN33, which contains a 2.5 kb genomic fragment including the hTR coding region (Feng, *et al.*, *supra*). The sequences of primers hTR+1 and hTR+445 used in PCR were 5'-GGGGAAGCTTTAATACGACTCACTATAGGGTTGCGGAGGGTGGGCCTG-3' and 5'-CCCCGGATCCTGCGCATGTGTGAGCCGAGTCCTGGG-3', respectively. hTR+1 contains the T7 promoter and a *Hind*III site at the 5' end. hTR+445 contains a *Bam*HI site and an engineered *Fsp*I site at +445 at the 5' end. PCR conditions were the following: 1 X Taq extender buffer (Stratagene), 0.5 uM primers, 1 ng template, 200 uM dNTPs (Pharmacia), 5U Taq extender (Stratagene), 5U Taq polymerase (Perkin-Elmer), 5 μg T4 gene 32 product (Boehringer Mannheim), 30 cycles at

94°C for 40 sec, 58°C for 20 sec and 72°C for 60 sec. The resulting clone, phTR+1, contained hTR downstream of the T7 promoter as confirmed by sequencing both strands of the inserted DNA by the dideoxy-mediated chain termination method as per the manufacturer's instructions (U.S. Biochemical).

phTR170, phTR180 and phTR190 were constructed by replacing a 114 bp *Xba*I-*Bsp*E1 fragment in phTR+1 by the same fragments (generated by PCR) containing 10 base pair mutations spanning positions 170-179, 180-189 or 190-199 of hTR respectively. phTR+1 digested with *Hind*III and *Bam*HI was used as a template in PCR. The 177 bp PCR fragment was digested with *Xba*I and *Bsp*E1 and the resulting 114 bp fragment cloned into the phTR+1 *Xba*I and *Bsp*E1 restriction sites. PCR conditions were as described for phTR+1 except 10 ng of the template fragment was used and the cycling conditions were the following: 5 cycles at 94°C 40 sec, 54°C 20 sec, 72°C 60 sec, followed by 25 cycles 94°C 40 sec, 60°C 20 sec, 72°C 60 sec. Primers hTR170 and TRC31 were used in PCR for constructing phTR170. The sequences of hTR170 and TRC31 are
5'-GGGGTCTAGAGCAAAGTTTTTTACACAGCTGCTGGCCCGTTC-3' and
5'-CCGAGAGACCCGCGGCTGACAGAG-3', respectively. phTR180 and phTR190 were constructed in a manner similar to phTR170, using primers hTR180 and hTR190, respectively, instead of hTR170. The sequences of hTR180 and hTR190 are
5'-GGGGTCTAGAGCAAACAAAAAATGTGTCGACGACCCCCGTTCGCCTCCCGG-3'
and
5'-GGGGTCTAGAGCAAACAAAAAATGTCAGCTGCTGGGGGCAAGCGGTCCCGGGGACC
TGCG-3', respectively. The resulting clones, phTR170, phTR180 and phTR190, contained the expected substitutions within the inserted *Xba*I/*Bsp*EI fragment, as confirmed by sequencing the inserted DNA.

In Figure 2, reconstituted telomerase was re-programmed to synthesize mutant telomere repeats and the

activity of the reconstituted telomerase with telmerase RNA mutations was assayed in the absence or presence of ATP as indicated: no RNA, lane 1; hTR1-557, lanes 2 and 3; hTR1-557 with a 17 base insertion at position 176 (hTR+17), lanes 4 and 5; hTR1-557 with a modified template (C_3A_3) encoding T_3G_3 repeats (hTR-C_3A_3), lanes 6 and 7; hTR1-557 with a modified template (C_4A_2) encoding T_2G_4 repeats (hTR-C_4A_2), lanes 8 and 9. As indicated, different C-strand oligonucleotides were used in the PCR assay to detect the appropriate telomerase elongation products, and 3 pmoles of RNA were added to each reaction. The gel was exposed to film for 4 days.

In Figure 3, telomerase was reconstituted with hTR of various sizes as indicated: no RNA, lane 1; hTR1-159 (159 nt), lane 2; hTR1-169 (169 nt), lane 3; hTR1-182 (182 nt), lane 4; hTR1-203 (203 nt), lane 5; hTR1-273 (273 nt), lane 6; hTR1-445 (445 nt), lane 7; hTR 44-184 (140 nt), lane 8; hTR 44-204 (160 nt), lane 9; hTR1-445 (445 nt), lane 10. Each reaction in lanes 2-7 included 2.5 pmoles of RNA, and 3 pmoles of RNA were added to reactions shown in lanes 8-10. Lanes 1-7 were exposed for 2 days and lanes 8-10 for 5 days.

Example 5

Preparation of RNAs

RNAs used in reconstitution were *in vitro* transcribed with SP6 or T7 RNA polymerase (Stratagene) using pGEM33 (encoding wild-type hTR plus downstream sequences-total length 557 nt-hTR1-557) digested with *Eco*RV, pGEM34 (encoding hTR1-557 with a C_3A_3-containing template), pGEM36 (encoding hTR1-557 with a C_4A_2-containing template) or pGEM38 (encoding hTR1-557 with a 17 bp insertion at residue 176) digested with *Eco*RV (Figures 1 and 2). The RNAs used in reconstitution contained 5′ (34 nt) and 3′ (41nt) flanking RNA from the pGEM vector which does not encode the

telomerase RNA. The RNAs made from phTR+1, phTR170, phTR180 and phTR190 contained only hTR sequences. The hTR44-170, hTR44-184 and hTR44-204 hTRs were made using DNA fragments generated by PCR. For all three, the 5' primer was T7hs48 (5'-TTCTAATACGACTCACTATAGGTCTAACCCTAACTGAGAAGG-3'). For hTR44-170, the 3' primer was R3C (5'-GTTTGCTCTAGAATGAACGGTGGAAG-3'). For hTR44-184, the 3' primer was ha188 (5'-AGCTGACATTTTTTGTTTGCTC-3'). For hTR44-204, the 3' primer was R7 (5'-GGAGGGGCGAACGGGCCAGCA-3'). Standard *in vitro* transcription reaction conditions recommended by the RNA polymerase manufacturer were used. The RNAs were either gel purified or the transcription reactions treated with 3U RNase-free DNase (Pharmacia) per μg of DNA for 10 min. The RNA concentrations were determined by specific activity determination of RNA synthesized with radionucleotides. The integrity and size of the RNAs were determined by Northern analysis or staining with ethidium bromide. Size of the RNAs are the following, with the actual number of residues of hTR and the enzyme used in parentheses: hTR1-557, 630 nt (*Eco*RV 557) for all hTRs made from pGEM based vectors (+17 nt for hTR+17). Sizes of the RNAs made from pUC119 based plasmids (phTR+1, phTR170, phTR180, phTR190) were the following: hTR1-159 (*Xba*I 159), hTR1-169 (*Bbv*I 169), hTR1-182 (*Pvu*II 182), hTR1-203(*Sma*I 203), hTR1-273 (*Bsp*E1 273) and hTR1-445 (*Fsp*I 445). The *Fsp*I site at position 445 was created by site-directed mutagenesis. The TGCAGT spanning nucleotides 443 to 448 was altered to TGCGCA which is cut by *Fsp*I. This construct was cloned into the plasmid pUC119. *Tetrahymena* telomerase RNA used as a control was *in vitro* transcribed as previously described (Autexier and Greider (1994) *Genes & Dev.* *8*:563-575). The 5S and 16S, 23S *E. coli* rRNAs were from Boehringer Mannheim and Sigma, respectively. Mouse

RNase P RNA and mouse telomerase RNA were a gift of Maria Blasco.

In Figure 4, telomerase was reconstituted with hTR of various sizes and sequence as indicated: mock-treated telomerase, lane 1; no RNA, lane 2; hTR1-159 (159 nt), lane 3; hTR1-169 (169 nt), lane 4; hTR1-182 (182 nt), lane 5; hTR170* (445 nt), lane 6; hTR180* (445 nt), lane 7; hTR190* (445nt), lane 8; hTR1-445 (445 nt), lane 9. The reactions shown in lanes 3-9 had 2.5 pmoles of RNA added to them. The gel was exposed to X-ray film for 2 days, except for lane 1, which was exposed for 18 hours.

Figure 5 is a linear representation of full-length hTR. The schematic includes the template region (white box) and positions of several restriction sites present in the gene encoding hTR. The *Fsp*I site was engineered into the gene. The 5' and 3' deletions and substitutions in hTR are indicated (stippled boxes), along with the relative activities these RNAs restore when added back to MNase-treated extract. The size of the transcribed RNAs are also indicated. For comparison, activity of hTR+17, which has a 17 nucleotide insertion at position 176 in hTR1-557 is included. The transcribed RNA in this case includes sequences downstream of hTR, plus vector sequences 5' and 3' to hTR.

Originally, the hTR sequence shown in Figure 7 was used to generate some of the reagents for the hTR reconstitution assays. The actual hTR sequence, discovered at Cold Spring Harbor Laboratory, is shown in Figure 6.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described specifically herein. Such equivalents are intended to be encompassed in the scope of the following claims.

Claims

We claim:

1. Isolated DNA sequences of hTR selected from the group consisting of:
   a) nucleotides 44-204 of hTR;
   b) nucleotides 1-203, 1-273, or 1-418 of hTR; and
   c) DNA encompassing nucleotides 44-204 and sequential deoxyribonucleotides but shorter than 1-445 of hTR.

2. Isolated RNA encoded by DNA of Claim 1.

3. Isolated DNA identical to or sharing the same biochemical and biological function of the DNA encoding the RNA of Claim 1.

4. A DNA sequence which hybridizes under high stringency conditions to the RNA according to Claim 2.

5. An RNA sequence transcribed from or complementary to the DNA sequence of Claim 3.

6. An RNA sequence transcribed from or complementary to the DNA sequence of Claim 4.

7. RNA of Claim 1 combined with endogenous or exogenous vertebrate telomerase protein.

8. A method of detecting telomerase activity with a DNA or RNA essential oligonucleotide in a cell, tissue or fluid sample comprising:
   (a) preparing the sample so that the DNA or the RNA will hybridize to any telomerase RNA in the sample;

(b) contacting the sample with the DNA or RNA so that hybridization occurs; and

(c) detecting any hybridization

wherein if hybridization occurs, active telomerase is present in the sample.

9. The method of Claim 8, wherein the amount of hybridization is measured and indicates the amount of telomerase in the sample.

10. A truncated vertebrate telomerase molecule comprising RNA encoded by isolated DNA of Claim 1 and the vertebrate telomerase protein.

11. A recombinant vertebrate telomerase molecule comprising a synthesized vertebrate telomerase RNA component combined with vertebrate telomerase protein.

12. A recombinant vertebrate telomerase molecule comprising RNA encoded by isolated DNA of Claim 1 and the vertebrate telomerase protein.

13. A pharmaceutical compound for increasing the amount of active telomerase in an individual comprising administering to the individual a therapeutically effective amount of the truncated vertebrate telomerase molecule of Claim 10.

14. A pharmaceutical compound for increasing the amount of active telomerase in an individual comprising administering to the individual a therapeutically effective amount of a recombinant vertebrate telomerase molecule.

15. A method of treating an individual in need of

telomerase by administering a therapeutically effective amount of truncated telomerase.

16. A method of treating an individual in need of telomerase by administering a therapeutically effective amount of DNA encoding recombinant telomerase.

17. A method of treating an individual in need of an inhibitor of telomerase by administering a therapeutically effective amount of the RNA encoded by the DNA of Claim 1.

18. A pharmaceutical compound for decreasing the amount of active telomerase in an individual comprising administering to the individual a therapeutically effective amount of an inhibitor of vertebrate telomerase.

19. A method of treating an individual in need of an inhibitor of telomerase by administering a therapeutically effective amount of the pharmaceutical compound of Claim 18.

20. A method for screening agents which inhibit, prevent, or stimulate telomerase activity comprising the steps of:

(a) contacting the potential agent with truncated or recombinant telomerase under conditions wherein telomerase is active; and

(b) determining whether the activity of telomerase is decreased or increased

whereby if the telomerase activity is decreased, the agent is identified as a telomerase inhibitor and, if the telomerase activity is increased, the agent is

identified as a telomerase stimulator.

21. A transgenic eukaryotic cell or organism containing the DNA sequence of Claim 1 or a sequence complementary to said sequence.

22. A transgenic prokaryotic cell containing the DNA sequence of Claim 1 or a sequence complementary to said sequence.

23. A process for producing a recombinant telomerase molecule comprising:

(a) producing an expression vector which includes DNA which encodes a recombinant telomerase molecule;

(b) transfecting or infecting a host cell with the vector; and

(c) culturing the transfected or infected cell line to produce the encoded recombinant telomerase molecule.

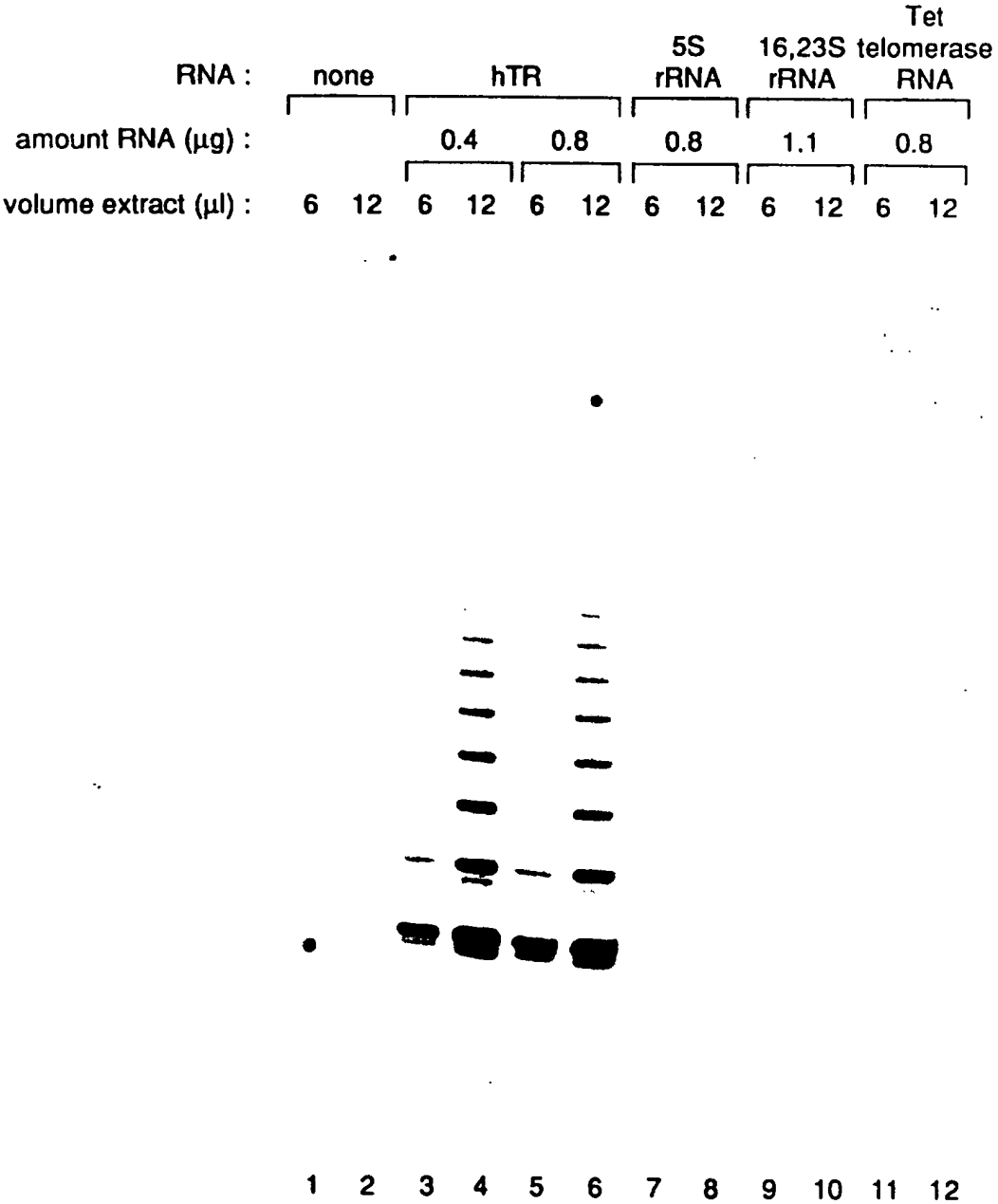
RNA :
none
hTR
5S rRNA
16,23S rRNA
Tet telomerase RNA
amount RNA (µg) :
0.4
0.8
0.8
1.1
0.8
volume extract (µl) :
6 12 6 12 6 12 6 12 6 12 6 12
1 2 3 4 5 6 7 8 9 10 11 12

FIG.1

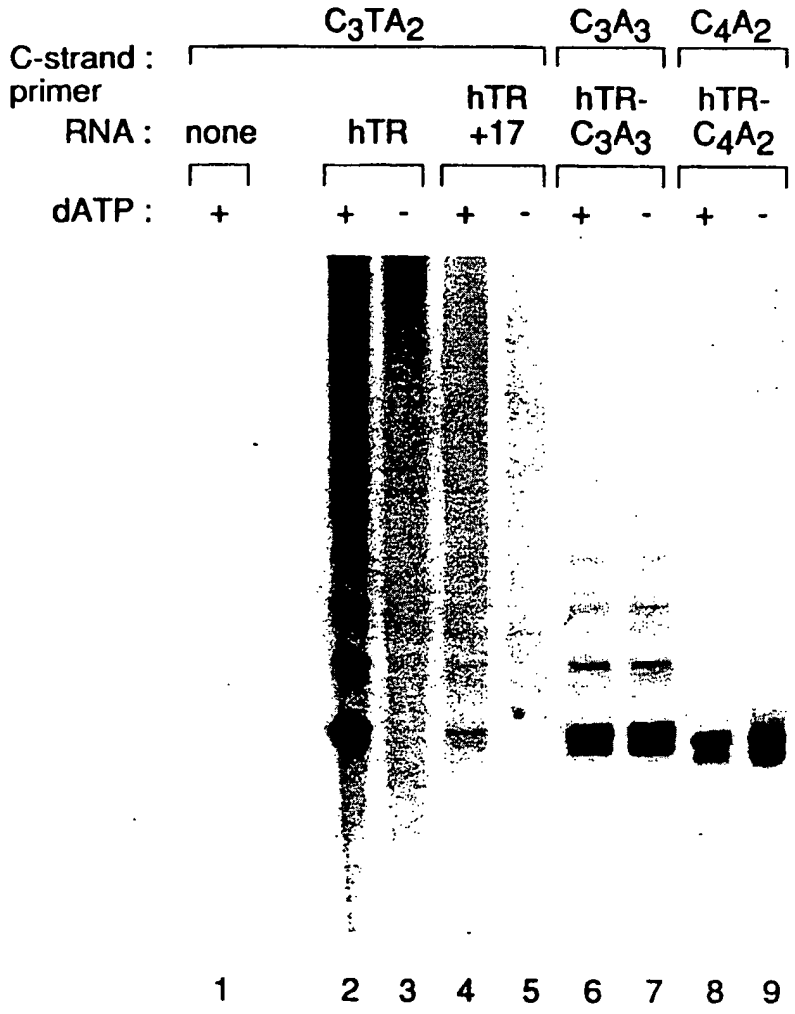
C3TA2
C3A3
C4A2
C-strand :
primer
RNA :
none
hTR
hTR +17
hTR-C3A3
hTR-C4A2
dATP :
+
+ - + - + - + -
1 2 3 4 5 6 7 8 9

FIG.2

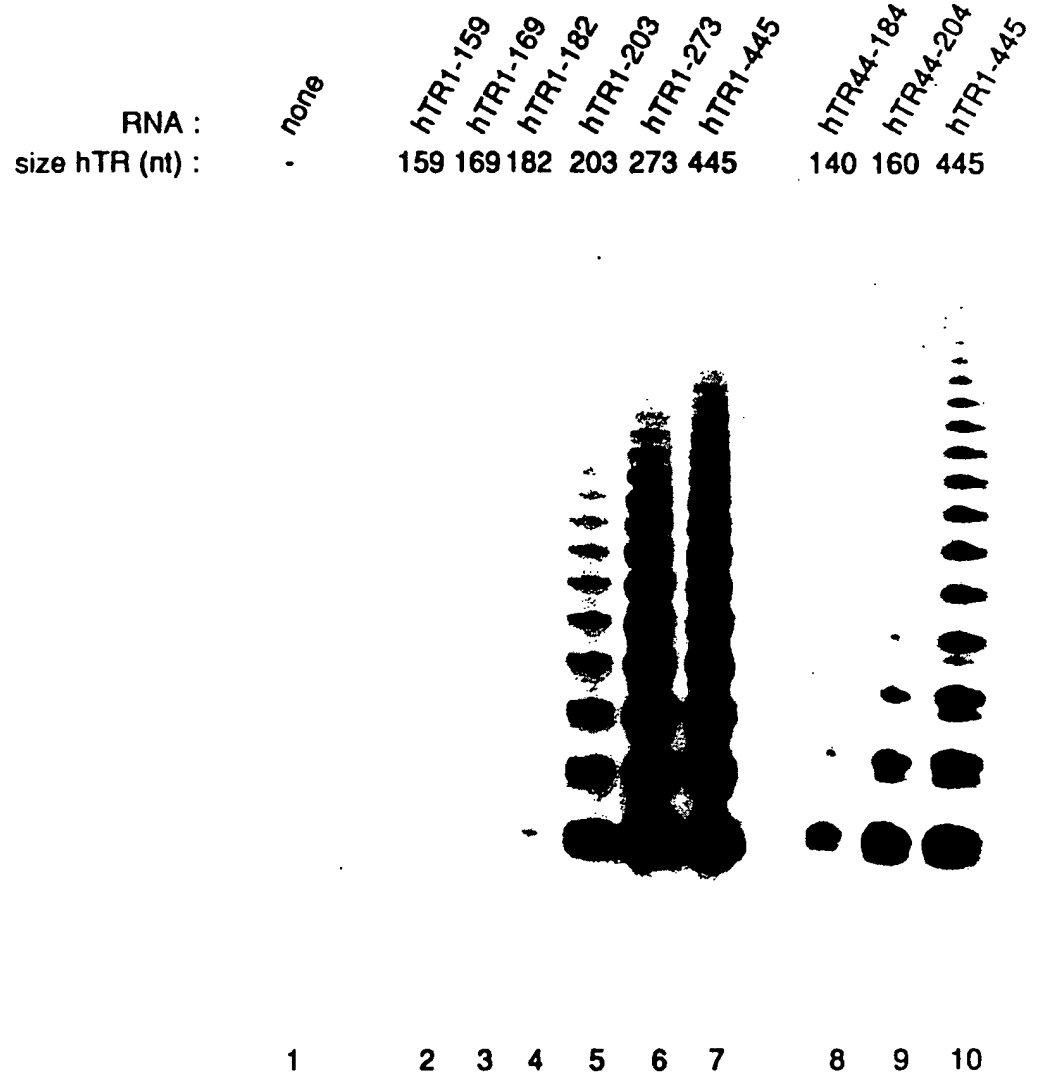
RNA :
size hTR (nt) :
none
-
hTR1-159
159
hTR1-169
169
hTR1-182
182
hTR1-203
203
hTR1-273
273
hTR1-445
445
hTR44-184
140
hTR44-204
160
hTR1-445
445
1
2
3
4
5
6
7
8
9
10

FIG.3

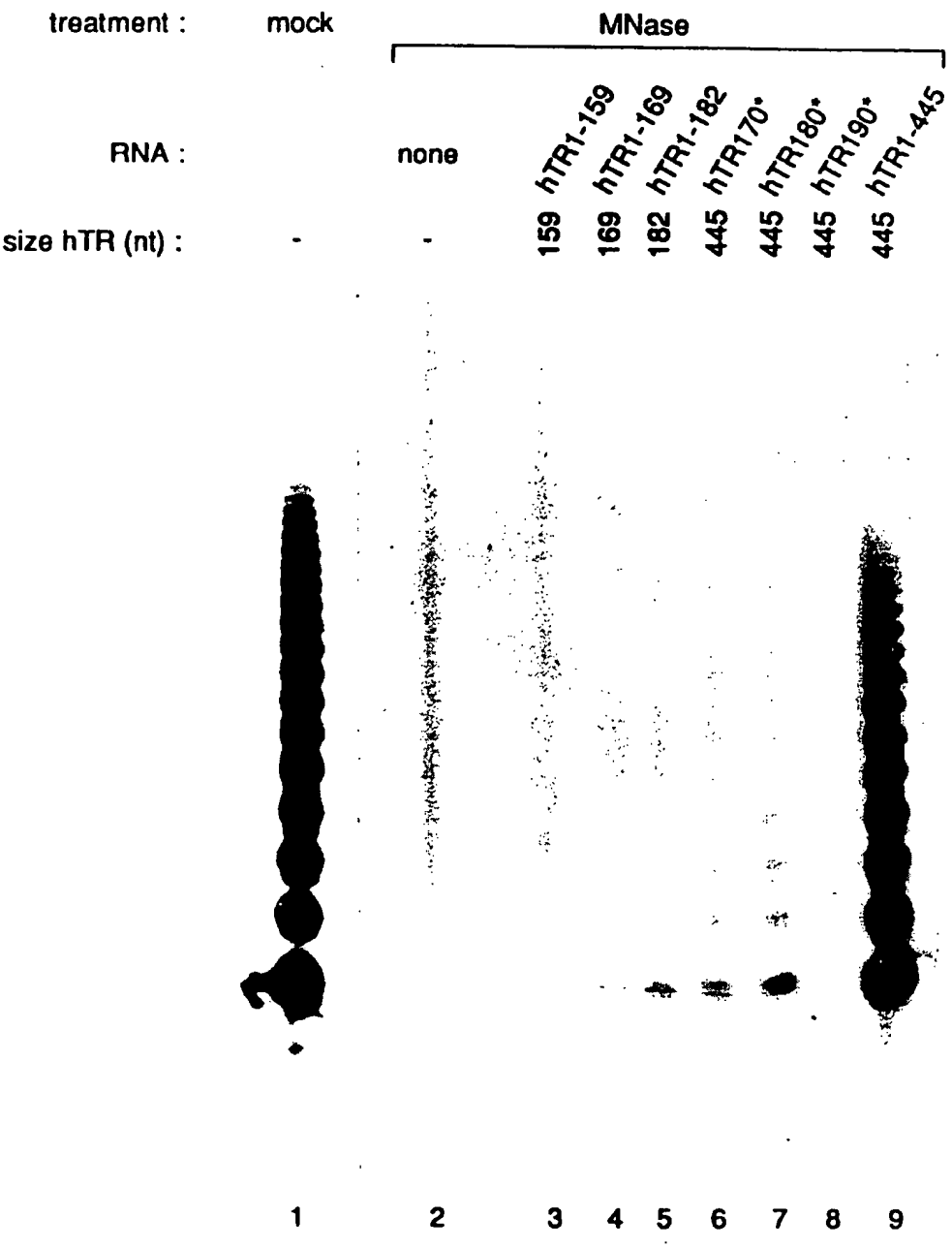
treatment :
mock
MNase
RNA :
none
hTR1-159
hTR1-169
hTR1-182
hTR170*
hTR180*
hTR190*
hTR1-445
size hTR (nt) :
-
-
159
169
182
445
445
445
445
1
2
3
4
5
6
7
8
9

FIG.4

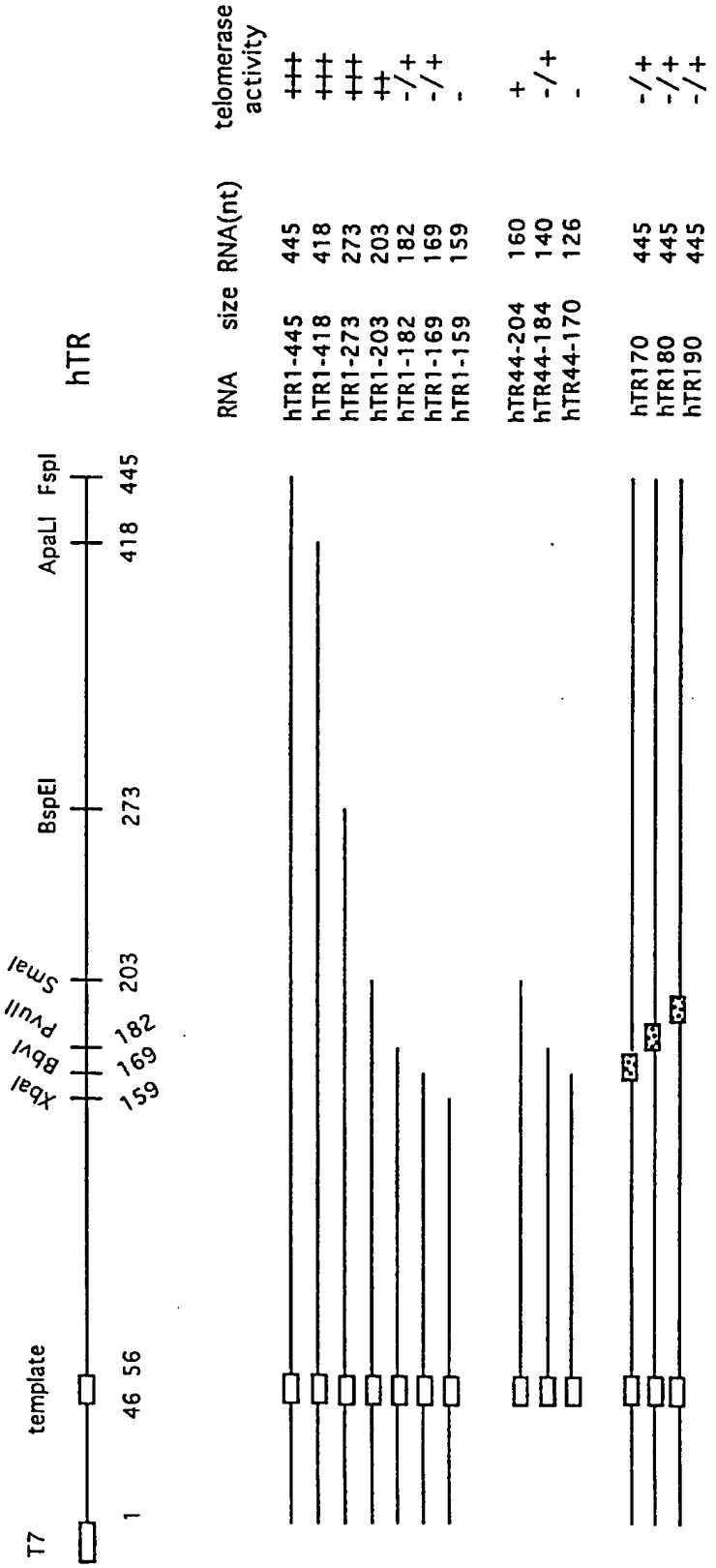
hTR
T7
template
1
46 56
XbaI
BbvI
PvuII
SmaI
159
169
182
203
BspEI
273
ApaLI
FspI
418
445
RNA size RNA(nt)
telomerase activity
hTR1-445 445 +++
hTR1-418 418 +++
hTR1-273 273 +++
hTR1-203 203 ++
hTR1-182 182 -/+
hTR1-169 169 -/+
hTR1-159 159 -
hTR44-204 160 +
hTR44-184 140 -/+
hTR44-170 126 -
hTR170 445 -/+
hTR180 445 -/+
hTR190 445 -/+

FIGURE 5

```
                                        GGGTTG CGGAGGGTGG     16

GCCTGGGAGG GGTGGTGGCC ATTTTTTGTC TAACCCTAAC                   56

TGAGAAGGGC GTAGGCGCCG TGCTTTTGCT CCCCGCGCGC                   96

TGTTTTTCTC GCTGACTTTC AGCGGGCGGA AAAGCCTCGG                   136

CCTGCCGCCT TCCACCGTTC ATTCTAGAGC AAACAAAAAA                   176
                          XbaI          BbvI

TGTCAGCTGC TGGCCCGTTC GCCTCCCGGG GACCTGCGGC                   216
     PvuII                      SmaI

GGGTCGCCTG CCCAGCCCCC GAACCCCGCC TGGAGCCGCG                   256

GTCGGCCCGG GGCTTCTCCG GAGGCACCCA CTGCCACCGC                   296
                    BspEI

GAAGAGTTGG GCTCTGTCAG CCGCGGGTCT CTCGGGGGCG                   336

AGGGCGAGGT TCACCGTTTC AGGCCGCAGG AAGAGGAACG                   376

GAGCGAGTCC CGCCGCGGCG CGATTCCCTG AGCTGTGGGA                   416

CGTGCACCCA GGACTCGGCT CACACATGCA GT                           448
 ApaLI                          FspI
```

FIGURE 6

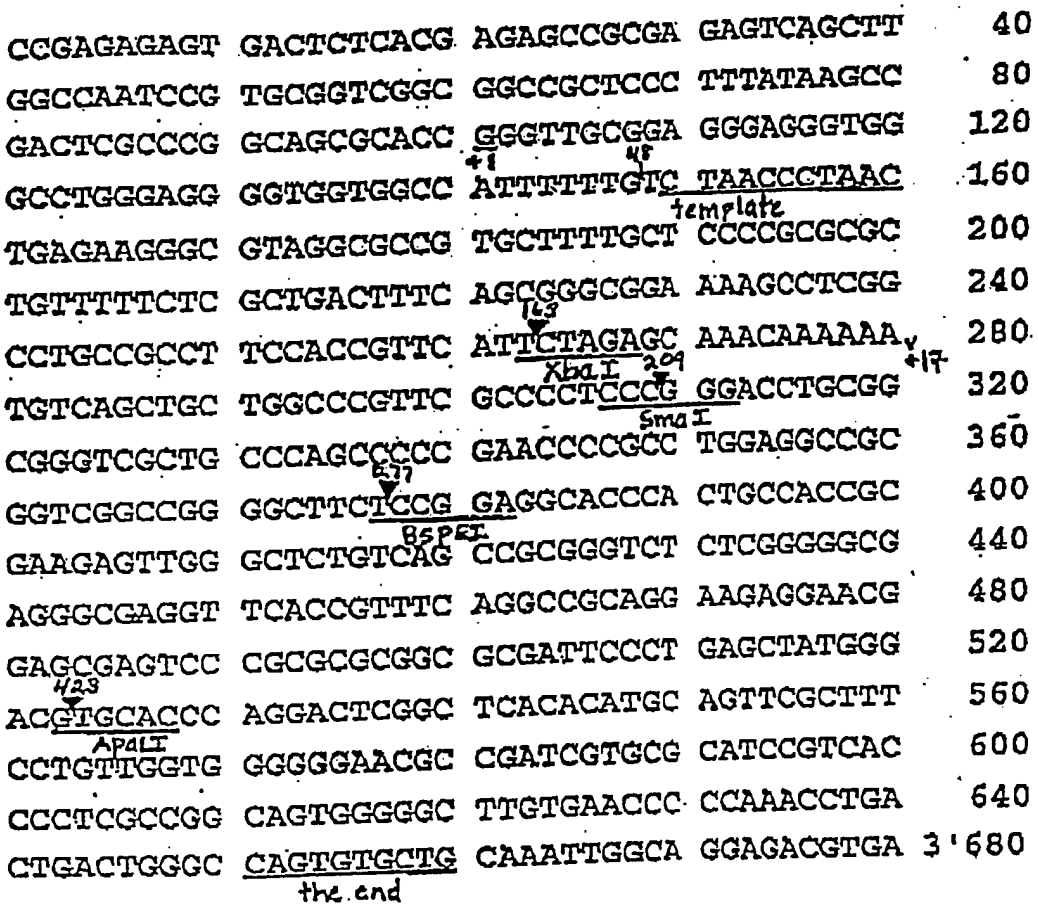
CCGAGAGAGT GACTCTCACG AGAGCCGCGA GAGTCAGCTT 40
GGCCAATCCG TGCGGTCGGC GGCCGCTCCC TTTATAAGCC 80
GACTCGCCCG GCAGCGCACC GGGTTGCGGA GGGAGGGTGG 120
+1
GCCTGGGAGG GGTGGTGGCC ATTTTTTTGTC TAACCCTAAC 160
45
template
TGAGAAGGGC GTAGGCGCCG TGCTTTTGCT CCCCGCGCGC 200
TGTTTTTCTC GCTGACTTTC AGCGGGCGGA AAAGCCTCGG 240
143
CCTGCCGCCT TCCACCGTTC ATTCTAGAGC AAACAAAAAA 280
XbaI 209 +17
TGTCAGCTGC TGGCCCGTTC GCCCCTCCCG GGACCTGCGG 320
SmaI
CGGGTCGCTG CCCAGCCCCC GAACCCCGCC TGGAGGCCGC 360
237
GGTCGGCCGG GGCTTCTCCG GAGGCACCCA CTGCCACCGC 400
BSPEI
GAAGAGTTGG GCTCTGTCAG CCGCGGGTCT CTCGGGGGCG 440
AGGGCGAGGT TCACCGTTTC AGGCCGCAGG AAGAGGAACG 480
GAGCGAGTCC CGCGCGCGGC GCGATTCCCT GAGCTATGGG 520
423
ACGTGCACCC AGGACTCGGC TCACACATGC AGTTCGCTTT 560
ApaLI
CCTGTTGGTG GGGGGAACGC CGATCGTGCG CATCCGTCAC 600
CCCTCGCCGG CAGTGGGGGC TTGTGAACCC CCAAACCTGA 640
CTGACTGGGC CAGTGTGCTG CAAATTGGCA GGAGACGTGA 3'680
the end

Figure 7

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/09517

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : Please See Extra Sheet.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN, BIOSIS, MEDLINE, CANCERLIT, EMBASE, CAPLUS, APS
search terms: telomerase, antisense, oligonucleotides, therapy, DNA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	WO 93/23572 A1 (GERON CORPORATION) 25 November 1993 (25.11.93), entire patent, specifically pages 17-28.	8-9, 11, 14, 16, 18, 20 --- 1-7, 10, 12-13, 15, 17, 19, 21-23
X --- Y	WO 95/13382 A1 (GERON CORPORATION) 18 May 1995 (18.05.95), see entire patent, especially pages 17-36.	8-9, 11, 14, 16, 18, 20 --- 1-7, 10, 12-13, 15, 17, 19, 21-23
Y, P	FENG et al. The RNA Component of Human Telomerase. Science. 01 September 1995, Vol. 269, pages 1236-1241, see entire document.	1-7, 10-12, 16-19, 23

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 20 AUGUST 1996	Date of mailing of the international search report 04 SEP 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer KAREN HAUDA Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/09517

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ROMERO et al. A Conserved Secondary Structure for Telomerase RNA. Cell. 18 October 1991, Vol. 67, No. 2, pages 343-353, see entire document.	1-7, 10-12, 16-19, 23
Y, P	EP 0,666,313 A2 (IOWA STATE UNIVERSITY RESEARCH FOUNDATION, INC.) 09 August 1995, entire patent.	1-7, 10-19, 21-23
Y	GREIDER et al. The Telomere Terminal Transferase of Tetrahymena Is a Ribonucleoprotein Enzyme with Two Kinds of Primer Specificity. Cell. 24 December 1987, Vol. 51, pages 887-898, see entire document.	1-7, 10-19, 21-23

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/09517

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C12N 5/00, 15/00, 15/63, 15/79; C07H 21/00; A61K 48/00, 38/00, 38/16, 38/43

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/ 320.1, 240.2, 6, 69.1 172.3, 7.2; 530/ 350, 828; 514/44; 935/62, 52, 55, 56, 34, 66, 70, 71, 65; 536/23.1, 23.5, 24.5; 424/93.2

B. FIELDS SEARCHED
Minimum documentation searched
Classification System: U.S.

435/ 320.1, 240.2, 6, 69.1 172.3, 7.2; 530/ 350, 828; 514/44; 935/62, 52, 55, 56, 34, 66, 70, 71, 65; 536/23.1, 23.5, 24.5; 424/93.2